



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00		A1	(11) International Publication Number: WO 92/22637 (43) International Publication Date: 23 December 1992 (23.12.92)
(21) International Application Number: PCT/US92/04988 (22) International Filing Date: 16 June 1992 (16.06.92) (30) Priority data: 716,264 17 June 1991 (17.06.91) US		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>	
(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 8717 Grovemont Circle, P.O. Box 6009, Gaithersburg, MD 20677 (US). (72) Inventors: DISORBO, Dennis, M. ; 90 Heath Street, Buffalo, NY 14214 (US). JAYME, David, W. ; 907 Carter Creek Drive, Grand Island, NY 14072 (US). (74) Agent: ESMOND, Robert, W.; Sterne, Kessler, Goldstein & Fox, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).			
(54) Title: MEDIA CONCENTRATE TECHNOLOGY			
(57) Abstract <p>The present invention relates to a method of subgrouping media formulations into stable, compatible components that can then be solubilized at high concentrations (10x to 100x). Concentrated solutions of the ingredient containing subgroups can be used to prepare media formulations. Concentrated culture media formulations (2-10x) or 1x cell culture media can be prepared according to the present invention by mixing a sufficient amount of the concentrated subgroup solutions with a sufficient amount of a diluent (water, buffer, etc.). The present invention further relates to stabilization of glutamine by complexing or mixing glutamine with divalent metal cations in solution.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		

MEDIA CONCENTRATE TECHNOLOGY

Field of the Invention

The present invention relates to the field of cell culture media and specifically to media ingredients subgrouped selectively into concentrated, compatible formulations. Such concentrated formulations are stable and can be used to prepare cell culture media.

Background of the Invention

Cell culture medium provides the nutrients necessary to maintain and grow cells in a controlled, artificial and *in vitro* environment. Characteristics and compositions of the cell culture media vary depending on the particular cellular requirements. Important parameters include osmolarity, pH, and nutrient formulations.

Media formulations have been used to grow a number of cell types including animal, plant and bacterial cells. Cells grown in culture media catabolize available nutrients and produce useful biological substances such as monoclonal antibodies, hormones, growth factors and the like. Such products have therapeutic applications and, with the advent of

-2-

recombinant DNA technology, cells can be engineered to produce large quantities of these products. Thus, the ability to grow cells in vitro is not only important for the study of cell physiology, it is necessary for the production of useful substances which may not otherwise be obtained by cost-effective means.

Cell culture media formulations have been well documented in the literature and a number of media are commercially available. Typical nutrients in cell culture media include amino acids, salts, vitamins, trace metals, sugars, lipids and nucleic acids. Often, particularly in complex media compositions, stability problems result in toxic products and/or lower effective concentrations of required nutrients, thereby limiting the functional life-span of the culture media. For instance, glutamine is a constituent of almost all media that are used in culturing of mammalian cells in vitro. Glutamine decomposes spontaneously into pyrrolidone carboxylic acid and ammonia. The rate of degradation can be influenced by pH and ionic conditions but in cell culture media, formation of these breakdown products cannot be avoided (Tritsch et al., Exp. Cell Research 28:360-364(1962)).

Wang et al. (In Vitro 14:(8):715-722 (1978)) have shown that photoproducts such as hydrogen peroxide, which are lethal to cells, are produced in Dulbecco's Modified Eagle's Medium (DMEM). Riboflavin and tryptophan or tyrosine are components necessary for formation of hydrogen peroxide during light exposure. Since most mammalian culture media contain riboflavin, tyrosine and tryptophan, toxic photoproducts are likely produced in most cell culture media.

-3-

To avoid these problems, researchers make media on an "as needed" basis, and avoid long term storage of the culture media. Commercially available media, typically in dry powder form, serves as a convenient alternative to making the media from scratch, i.e., adding each nutrient individually, and also avoids some of the stability problems associated with liquid media. However, only a limited number of commercial culture media are available, except for those custom formulations supplied by the manufacturer.

Although dry powder media formulations may increase the shelf-life of some media, there are a number of problems associated with dry powdered media, especially in large scale application. Production of large media volumes requires storage facilities for the dry powder media, not to mention the specialized media kitchens necessary to mix and weigh the nutrient components. Due to the corrosive nature of dry powder media, mixing tanks must be periodically replaced.

There exists a need to lower the cost of production of biological substances. Efficient and cost effective methods to stabilize liquid cell culture media as well as the development of convenient methods to produce 1x media formulations would be an important development in the field of cell culture media technology.

Summary of the Invention

The present invention relates to a method of subgrouping media formulations into stable, compatible components that can then be solubilized at high concentrations (10x to 100x) in solution containing, for example, water, acid, alkali or alcohol, depending

-4-

on the subgroup. Any media formation can be divided into one or more of the six subgroups of the present invention. Specific subgroups include an acid soluble subgroup, a glutamine containing subgroup, an alkali soluble subgroup, an alcohol soluble subgroup, a weak acid-base soluble subgroup and a supplement-containing subgroup.

According to the present invention, one of ordinary skill in the art may divide ingredients of a cell culture media into one of the subgroups of the present invention based on the physical and chemical properties (physicochemical properties) of each ingredient. Ingredients of culture media typically include amino acids, salts, vitamins, trace metals, sugars, lipids, nucleic acids and the like. These ingredients have different solubility and stability characteristics. Based on these characteristics, a classification scheme has been developed to permit the compatible subgrouping of media ingredients.

The invention thus relates to a method of subgrouping compatible media ingredients. Media ingredients, subgrouped in this manner, are stable and remain soluble even at high concentrations. Such concentrated solutions of the ingredient containing subgroups can then be used to prepare diluted media formulations suitable for growing or maintaining cells. Concentrated culture media formulations (2-10x) or 1x cell culture media can be prepared according to the present invention by admixing sufficient amount of the concentrated subgroup solutions with a sufficient amount of a diluent (water, buffer, etc.). Thus, the present invention is directed to a method of preparing a cell culture media comprising:

-5-

- (a) dividing the compatible ingredients of a cell culture media into one or more subgroups, such subgroups comprising:
 - (i) an acid soluble subgroup,
 - (ii) a weak acid-base soluble subgroup,
 - (iii) a glutamine-containing subgroup,
 - (iv) an alcohol soluble subgroup,
 - (v) an alkali-soluble subgroup, and
 - (vi) a supplement-containing subgroup;
- (b) dissolving each of the subgroups of compatible ingredients in a carrier to give concentrated solutions of the subgroup in the respective carrier; and
- (c) admixing a sufficient amount of each of the subgroups obtained in step (b) with a sufficient amount of a diluent to produce the cell culture media.

Furthermore, the present invention is specifically directed to a method of preparing RPMI-1640 media comprising:

- (a) dividing the ingredients of RPMI-1640 media into
 - (i) an acid soluble subgroup,
 - (ii) a weak acid-base soluble subgroup, and
 - (iii) a glutamine-containing subgroup;
- (b) dissolving each of the subgroups of compatible ingredients in a carrier to give concentrated solutions of the subgroup in the respective carrier; and
- (c) admixing a sufficient amount of each of the subgroups obtained in step (b) with a sufficient amount of a diluent to produce the RPMI-1640 media.

-6-

This method can also be used to make DMEM media. That is, the ingredients of DMEM media can be divided into an acid soluble subgroup, a weak acid-base soluble subgroup and a glutamine-containing subgroup which are combined at some later time and diluted to give a 1x formulation.

The present invention further relates to stabilization of glutamine by complexing or mixing glutamine with divalent metal cations in solution. Thus, the present invention is also directed to a composition of matter comprising glutamine and a divalent cation in an amount effective to stabilize the glutamine.

Unexpectedly, the present invention provides for the preparation of media at dramatically reduced cost. The cost reductions are due to the following factors: The media concentrates of the present invention may be produced with much smaller production facilities since the large stir tanks required for 1x medias are not required. In addition, the media concentrates of the present invention may be prepared on an as needed basis using "just in time" production techniques which reduce inventory, storage and labor costs. The time required for the preparation and shipping of the media may be reduced from 6-8 weeks to as little as one day. In addition, the media concentrates of the present invention may be prepared without the use of ball milling machines since each of the subgrouped ingredients is readily soluble. In addition, the media subgroups of the present invention may be stored at room temperature which eliminates the need for refrigeration. In addition, the present invention allows for the preparation of concentrates which may be used to prepare very large quantities of 1x media (100,000 liters or greater) which require only one

-7-

quality control test compared to multiple quality control tests for multiple batches produced according to the prior art. Importantly, the media concentrates of the present invention are much more consistent between batches since the subgrouped components are more stable. Thus, the present invention is a great advance in the art of media formulation.

Brief Description of the Drawings

Figure 1 shows L-glutamine stabilization in solution. Various ratios of L-glutamine to divalent cations were tested and the stability of L-glutamine was determined.

Figure 2 shows the monitored pH and osmolarity over time of RPMI-1640 media prepared by the continuous admixture of a sufficient amount of concentrated subgroups with a sufficient amount of water to make a 1x media formulation.

Figure 3 shows the monitored level of L-glutamine and glucose over time. The samples were taken from a mixing chamber during the continuous admixture of concentrated subgroups with water.

Figure 4 shows a kit comprising large containers containing concentrated subgroups. The concentrated subgroups may be admixed and diluted to give a total of 10,000 liters of media.

Description of the Preferred Embodiments

In the description that follows, a number of terms conventionally used in the field of cell culture media are utilized extensively. In order to provide a clear and consistent understanding of the

specification and claims, and the scope to be given such terms, the following definitions are provided.

Ingredients. The term "ingredients" refer to any compound, whether of chemical or biological origin, that can be used in cell culture media to maintain or promote the growth or proliferation of cells. The terms "component", "nutrient" and "ingredient" can be used interchangeably and are all meant to refer to such compounds. Typical ingredients that are used in cell culture media include amino acids, salts, metals, sugars, lipids, nucleic acids, hormones, vitamins, fatty acids, proteins and the like. Other ingredients that promote or maintain growth of cells in vitro can be selected by those of skill in the art, in accordance with the particular need.

Cell Culture. By "cell culture" is meant cells or tissues that are maintained, cultured or grown in an artificial, in vitro environment.

Culture Vessel. Glass, plastic or metal containers of various sizes that can provide an aseptic environment for growing cells are termed "culture vessels."

Cell Culture Media. The phrases "cell culture media" or "culture media" or "media formulation" refer to a nutritive solution for culturing or growing cells. The ingredients that compose such media may vary depending on the type of cell to be cultured. In addition to nutrient composition, osmolarity and pH are considered important parameters of culture media.

Compatible Ingredients. Each ingredient used in cell culture media has unique physical and chemical characteristics. By "compatible ingredients" is meant those media nutrients which can be maintained in solution and form a "stable" combination. A solution

-9-

containing "compatible ingredients" is said to be "stable" when the ingredients do not degrade or decompose substantially into toxic compounds, or do not degrade or decompose substantially into compounds that can not be utilized or catabolized by the cell culture. Ingredients are also considered "stable" if degradation can not be detected or when degradation occurs at a slower rate when compared to decomposition of the same ingredient in a 1x cell culture media formulation. Glutamine, for example, in 1x media formulations, is known to degrade into pyrrolidone carboxylic acid and ammonia. Glutamine in combination with divalent cations are considered "compatible ingredients" since little or no decomposition can be detected over time.

Compatibility of media ingredients, in addition to stability measurements, are also determined by the "solubility" of the ingredients in solution. The term "solubility" or "soluble" refers to the ability of an ingredient to form a solution with other ingredients. Ingredients are thus compatible if they can be maintained in solution without forming a measurable or detectable precipitate. Thus, the term "compatible ingredients" as used herein refers to the combination of particular culture media ingredients which, when mixed in solution either as concentrated or 1x formulations, are "stable" and "soluble."

1x Formulation. A cell culture media is composed of a number of ingredients and these ingredients vary from media to media. A "1x formulation" is meant to refer to any aqueous solution that contains some or all ingredients found in a cell culture media. The "1x formulation" can refer to, for example, the cell culture media or to any subgroup of ingredients for

-10-

that media. The concentration of an ingredient in a 1x solution is about the same as the concentration of that ingredient found in the cell culture formulation used for maintaining or growing cells. Cell culture media used to grow cells is a 1x formulation by definition. When a number of ingredients are present (as in a subgroup of compatible ingredients), each ingredient in a 1x formulation has a concentration about equal to the concentration of those ingredients in a cell culture media. For example, RPMI 1640 culture media contains, among other ingredients, 0.2 g/l L-arginine, 0.05 g/l L-asparagine, and 0.02 g/l L-aspartic acid. A "1x formulation" of these amino acids, which are compatible ingredients according to the present invention, contains about the same concentrations of these ingredients in solution. Thus, when referring to a "1x formulation," it is intended that each ingredient in solution has the same or about the same concentration as that found in the cell culture media being described. The concentrations of media ingredients in a 1x formulation are well known to those of ordinary skill in the art, See Methods For Preparation of Media, Supplements and Substrate For Serum-Free Animal Cell Culture Alan R. Liss, N.Y. (1984), which is incorporated by reference herein in its entirety. The osmolarity and/or pH, however, may differ in a 1x formulation compared to the culture media, particularly when fewer ingredients are contained by the 1x formulation.

10x Formulation. A "10x formulation" refers to a solution wherein each ingredient in that solution is about 10 times more concentrated than the same ingredient in the cell culture media. RPMI 1640

media, for example, contains, among other things, 0.3 g/l L-glutamine. By definition, a "10x formulation" contains about 3.0 g/l glutamine. A "10x formulation" may contain a number of additional ingredients at a concentration about 10 times that found in the 1x culture media. As will be apparent, "25x formulation," "50x formulation" and "100x formulation" designate solutions that contain ingredients at about 25, 50 or 100 fold concentrations, respectively, as compared to a 1x cell culture media. Again, the osmolarity and pH of the media formulation and concentrated formulation may vary.

A. Media Concentrate Technology

The Media Concentrate Technology of the present invention is based on the subgrouping of media ingredients into stable, compatible components that can then be solubilized into concentrated formulations (10X-100X) by the dissolution in an appropriate quantity of a carrier such as water, aqueous acid, alcohol or aqueous alkali, depending on the particular subgroup. Such solutions are referred to herein as a "carrier". Under appropriate conditions, these subgroups can be reconstituted (admixed) to produce a concentrated (greater than 1x) cell culture media formulation, such as a 2x to 10x, or reconstituted directly to a 1x cell culture media formulation. The concentrate media technology of the present invention is related to the subgrouping of compatible components, production of liquid concentrates of such subgroups, and mixing sufficient amounts of these concentrated subgroups with a sufficient amount of a diluent to produce a desired media formulation.

-12-

Cell culture media formulations are made up of amino acids, salts, vitamins, trace metals, sugars, lipids, nucleic acids, etc. Each of these components has different solubility and stability properties. Amino acids, for example, (except for glutamine) are soluble and very stable under acidic conditions (pH of about 1.0) while vitamins (except for thiamine and ascorbic acid) are solubilized under dilute acid or alkaline conditions (pH of about >4.0). Based on the physical properties of media ingredients, a classification scheme was developed that permits the subgrouping of any media formulation which can be used to prepare stable liquid concentrates.

The present invention defines six subgroups in which compatible ingredients from any cell culture media can be divided. The ingredients of a given cell culture media formulation can be separated into at least one of the subgroups defined by the present invention. Each subgroup, containing the compatible media ingredients, can then be dissolved in a liquid carrier or maintained in dry form. The type of liquid carrier and the method used to dissolve the grouped ingredients into solution vary depending on the subgroup and can be determined by one of ordinary skill in the art with no more than routine experimentation.

Preferably, the solutions comprising the subgrouped ingredients are more concentrated than the concentration of the same ingredients in a 1x media formulation. The grouped ingredients are preferably 25 fold more concentrated (25x formulation) and most preferably 50 fold more concentrated (50x formulation). Higher concentrated formulations can be

-13-

made provided that the ingredients remain soluble and stable.

Once the media ingredients have been divided into the one or more of the six subgroups and prepared as separate concentrated solutions, an appropriate (sufficient) amount of each concentrate is combined with a diluent to produce a 1x media formulation. Typically, the diluent used is water but other solutions including aqueous buffers, aqueous saline solution, or other aqueous solutions containing particular media ingredients may be used according to the invention.

The culture media of the present invention is typically sterilized to prevent unwanted contamination of the cell culture media. Sterilization may be accomplished, for example, by pasteurization after admixing the concentrated ingredients to produce a sterile culture media. Alternatively, each concentrated subgroup may be sterilized and stored as a sterile solution. These sterile concentrates can then be mixed with a sterile diluent to produce a concentrated 1x sterile media formulation.

One of a number of commonly used sterilization techniques may be used to sterilize the concentrated subgroups or the media formulations of the present invention. Sterilization techniques will vary depending on the properties of the solutions to be sterilized. Typical sterilization techniques, according to the present invention, include heat and/or filter sterilization. Other types of sterilization will be readily apparent to one of ordinary skill in the art.

B. Type of Media

Any type of cell culture media can be made according to the present invention. Culture media formulations are well-known in the literature and a number are commercially available. New media formulations and known media formulations which have been modified can also be prepared according to the present invention. That is, the media ingredients can be divided into compatible subgroups as defined in the present invention. The subgroups of these ingredients can then be concentrated in solution and, if desired, sterilized.

Examples of cell culture media that can be prepared according to the present invention include, but are not limited to, DMEM, RPMI-1640, DMEM/F12, IMDM, MEM, M199, McCoy's 5A, and F12 Nutrient Mixture (GIBCO/BRL CATALOGUE & PREFERENCE GUIDE (1990)).

The present invention thus provides a means for making one or more concentrated solutions of compatible ingredients, which when sufficient amounts are mixed in combination with sufficient amounts of diluent, a cell culture media may be produced.

Any ingredient employed in a cell culture media can be divided into one or more of the six subgroups of the present invention. Although the list of ingredients described herein and contained by each subgroup may not be all-inclusive, one of skill in the art can easily determine, by knowing the physicochemical properties of the ingredient, which subgroup contains other ingredients compatible to that ingredient. Alternatively, if the properties of the new ingredient are not known, routine experimentation can be used to determine the preferred subgroup. For

-15-

example, each subgroup containing particular media ingredients as defined according to the present invention, can be supplemented with the new ingredient. The new ingredient will be compatible with a particular subgroup when the resultant concentrated solution is soluble and stable. By comparing experimental media preparations to control media not made according to the media concentrate technology, one of skill may further determine the preferred subgroup. Growth characteristics of cells, formation of toxic products, and shelf-life of the media may be used as indicators of compatible ingredients.

Particular ingredients that have unique physicochemical properties may, in some situations, not be compatible with the ingredients of any subgroup. In such a case, it may be necessary to maintain this component as a separate subgroup. Such ingredients may be added to the diluent in an appropriate concentration so that a desired 1x culture media (containing this ingredient) is produced when sufficient amounts of the concentrated subgroups are admixed with the diluent. Alternatively, the incompatible ingredient can be added as a separate concentrated solution. Thus, the present invention envisions the development of additional subgroups, other than those defined in the present invention, which stabilize and solubilize additional ingredients which may not be compatible with any subgroups defined herein.

It will be apparent from the list of ingredients designated for each subgroup described below that a number of ingredients can be included in more than one subgroup. When a particular ingredient is designated

as compatible with two or more subgroups, that ingredient can be included in one or more of these subgroups at various concentrations, as long as the total amount of that ingredient conforms to the final media formulation. The only limitation is that the amount of ingredient added does not adversely affect solubility or stability of the other ingredients in the particular subgroup. The number and types of subgroups necessary to prepare different media may vary according to media complexity and/or the physicochemical properties of the ingredients. The duplication of ingredients in more than one subgroup thus provides one of ordinary skill in the art with a versatile classification scheme to prepare any media.

C. Acid Soluble Subgroup

Media ingredients placed in the acid soluble subgroup comprise all amino acids (except glutamine), thiamine, ascorbic acid, ferric compounds, ferrous compounds, purines, glutathione and monobasic sodium phosphates. These components are soluble in acid (pH of about 0 to 1.0) and are very stable under these conditions.

Both D and/or L-amino acids may be included in the acid-soluble subgroup of the present invention. Typically, culture medium contain the L-amino acids. As required by the desired media formulation, one or more amino acids in any combination may be included in the acid-soluble subgroup, with the exception of glutamine. Glutamine is specifically included in the glutamine-containing subgroup. Thus, the amino acids that may be included in the acid-soluble subgroup of the present invention, depending on the requirements

of the desired culture media, include glycine, alanine, valine, leucine, isoleucine, serine, threonine, cysteine, cystine, methionine, asparagine, aspartic acid, glutamic acid, lysine, arginine, phenylalanine, tyrosine, proline, hydroxyproline, histidine, and tryptophan. Derivatives of amino acids may also be included in the acid-soluble group provided that the derivative is compatible with the other ingredients of this subgroup and with the maintenance or proliferation of the particular cell culture.

Other ingredients which may be included in the acid-soluble subgroup of the present invention include thiamine (vitamin B₁), ascorbic acid (vitamin C), purines (guanine and adenine) and glutathione. Derivatives of such compounds are well-known and may be included in the acid-soluble group, provided that the physicochemical properties have not been changed to the extent that the derivative is no longer compatible with the other ingredients included in this subgroup and with the maintenance or proliferation of the particular cell culture.

Ferric and ferrous compounds, if required by the media formulation, may be placed into this subgroup as well. Such ferric and ferrous compounds, that are stable and soluble in the presence of the other ingredients included in this subgroup, can easily be determined by one of skill in the art. Examples of such ferrous or ferric compounds include, but are not limited to, $Fe(NO_3)_3$, $FeSO_4$, and $FeCl_3$.

Any monobasic phosphate which is a component of the desired media formulation can be contained within the acid-soluble subgroup. Examples of typical

phosphates include, but are not limited to, monobasic phosphates such as NaH_2PO_4 and KH_2PO_4 .

The acid-soluble subgroup of the present invention may contain various combinations of the above-noted compatible ingredients, as required by the particular culture media. Thus, according to the classification scheme of the present invention, the ingredients found in the desired culture media are selected which are compatible in an acid solution. Once divided in this manner, these ingredients, typically in dry powder form (dehydrated), are dissolved in an acidic solution with a pH of about 0 to 1.0. Preferably, the resulting solution is concentrated (10x to 100x). That is, the ingredients in the acid solution are more concentrated than the same ingredients found in a 1x media formulation.

As will be apparent, the concentrated acid-soluble subgroup may vary slightly in composition, osmolarity and pH from preparation to preparation of the same media. Furthermore, the acid-soluble subgroup will also differ from one media to the next. This is true for the other subgroups as well. The present invention provides the means to produce a stable preparation containing acid-soluble media ingredients. Such a preparation can be maintained as a dry powder mix of ingredients but preferably are maintained as a concentrated solution. Typically, the concentrated solution comprising the acid soluble subgroup is sterilized by one of a number of well-known techniques.

D. Glutamine Containing Subgroup

Glutamine is considered an unstable component in liquid media (Tritsch, G.L. *et al.*, Experimental Cell Research **28**:360-364 (1962); Ozturk, S.S. *et al.*, Biotechnol. Prog. **6**:121-128 (1990)). In solution, glutamine decomposes to pyrrolidone carboxylic acid and ammonia. The advent of media concentrate technology according to the present invention provides a procedure for the stabilization of glutamine. Thus, cell culture media that contains glutamine can be made by preparing, in addition to other subgroups, a glutamine-containing subgroup.

According to the invention, glutamine (both D and L-forms) can be stabilized in solution by the addition of one or more divalent cations. Any divalent cation, such as Ca^{+2} , Mg^{+2} , Mn^{+2} , Z^{+2} , and Cu^{+2} can be used to stabilize glutamine. A number of readily available salts including, but not limited to, MgCl_2 , CaCl_2 , $\text{Ca}(\text{NO}_3)_2$, and MgSO_4 may supply these divalent cations. Thus, any compatible calcium salt, magnesium salt, and/or manganese salt may be included in this subgroup. Such salts are typical ingredients of almost any media. Therefore, the divalent salts of the media formulation should be included in this subgroup to stabilize glutamine. In situations where divalent cations are generally not ingredients of the desired culture media, divalent cations that do not adversely affect the growth or the maintenance of the cells to be grown should be added to stabilize glutamine in the glutamine-containing subgroups.

The only limitation, however, with regard to the glutamine-containing subgroup of the invention is that potassium salts such as potassium chloride cannot be

-20-

included in this group. Sodium and magnesium ions decrease the solubility of potassium ions. Additionally, metal salts of phosphate cannot be put into this group since Ca^{2+} forms an insoluble complex with phosphate, removing Ca^{2+} from the solution.

The amount of glutamine as required by the particular media is mixed with a sufficient amount of a divalent cation to stabilize glutamine. The ratio of glutamine to divalent cations in this subgroup is preferably about 2:1 (weight to weight) but may range from 2:1 to 2.9:1. The pH of such a solution is also important for stabilizing glutamine. The preferred pH may range from 4.0 to 6.0. The most preferred pH of the glutamine-divalent cation solution is about 5.5. Basic pHs tend to lower glutamine stability, and thus, if necessary, pH may be adjusted with an acid which is compatible with the glutamine containing subgroup, e.g. HCl.

When the ratio of glutamine to divalent cation is about 2:1, glutamine forms a stable soluble complex. This complex does not favor the breakdown of glutamine to pyrrolidone carboxylic acid and ammonia. The glutamine metal complex thus provides a means for supplying this essential nutrient in a stable, soluble liquid form.

The ingredients contained in the glutamine-containing subgroup may comprise glutamine, calcium salts, and/or magnesium salts. D-Ca pantothenate may also be included in the glutamine containing subgroup, as well as sodium chloride.

As will be evident, each culture medium prepared according to the present invention may have a preferred osmolarity range. Osmolarity will change, as will the final pH of the culture media, with the

-21-

amount of acid/base used for solubilization of ingredients in each subgroup. One of skill in the art can easily determine the amount of salt, e.g., sodium chloride, necessary to obtain the preferred final osmolarity of the desired culture media by taking into consideration the final pH and volume of each concentrated subgroup required to prepare the media formulation. Such parameters (pH of each subgroup and total salt content) can be easily determined by routine experimentation. For example, the pH of the ingredient containing subgroups can be measured by well-known techniques. The osmolarity of the 1x media formulation, prepared by mixing an appropriate volume of these concentrated subgroups allows one of ordinary skill in the art to determine the amount of sodium chloride needed, if any, to adjust the final osmolarity of the culture media. This amount of sodium chloride may be added to the glutamine-containing subgroup to achieve the proper final osmolarity. Most 1x media formulations have broad range requirements for final osmolarity and pH and therefore slight variations in subgroup pH and salt concentration are not critical.

E. Weak Acid and Base Soluble Subgroup

The media ingredients of the weak acid-weak base soluble subgroup (referred to herein as a weak acid-base soluble subgroup) consist of sugars, deoxyribose, ribose, nucleotides, water-soluble vitamins (except for thiamine, ascorbic acid and Ca-pantothenate) riboflavin, salts, e.g., potassium chloride and sodium chloride, trace metals, lipids, acetate salts,

-22-

phosphate salts, HEPES, phenol red, pyruvate salts, and buffers.

The pH of the weak acid-base subgroup may range from about 4.0-9.0. Dibasic sodium phosphate and some monobasic sodium phosphate may also be grouped here. The combination of dibasic and monobasic sodium phosphate provides a buffer system for this concentrated subgroup. Adjustment of pH to the solution of this subgroup is preferably made by the addition of sodium hydroxide or hydrochloric acid, as appropriate, but not potassium hydroxide.

The ingredients found in this subgroup are determined, as with all other subgroups, by the media composition. Thus, any combination of the above ingredients may be included in the weak acid-base soluble group.

Sugars that are included in this subgroup include any sugar that can be used as a media ingredient and is compatible in combination with one or more of the above ingredients. Such sugar compounds include glucose, galactose, maltose, mannose, etc., and derivatives thereof.

Sugars in this subgroup may also serve as a vehicle for solubilizing lipids, if lipids are included in this subgroup. Lipids can be solubilized in solution after being desiccated onto a sugar or salt molecule. Briefly, lipids are solubilized in a small volume of ethanol and then slowly dripped onto a dry powder of sugar/salt. The mixture is dried for 3 hours and then ball milled. The sugar-lipid or salt-lipid composition can then be prepared as a concentrate comprising the other ingredients of the weak acid-base subgroup.

-23-

Any lipid and/or fat soluble ingredient including, but not limited to, linoleic acid, lipoic acid, cholesterol, calciferol, vitamin A, 2-mercaptopropanoic acid, tween 80, menadione and vitamin E can be desiccated onto a salt or sugar and thus included in this subgroup.

Any deoxyribose, ribose or nucleotide may be included in the weak acid and base soluble subgroup according to the present invention. These compounds and their derivatives are well-known. Examples include adenosine and adenosine triphosphate. Again, the only limitation is that the ingredient must be compatible with the other ingredients found in this subgroup.

Vitamins in the weak acid-base subgroup may include all water-soluble vitamins and their derivatives except thiamine, ascorbic acid and Ca-pantothenate. Such water-soluble vitamins that may be included in the weak acid-base soluble group include folic acid, biotin, pyridoxine, nicotinic acid, riboflavin, and the like.

Trace metals, used in media formulations, are also included in this subgroup. Examples of trace metals include CuSO_4 , Na_2SeO_3 , ZnSO_4 , and CoCl_2 . Trace amounts of other metals may also be included in this group. The selection of appropriate trace metals and the amounts employed may be readily determined by one of ordinary skill in the art.

Potassium chloride, dibasic sodium phosphate, sodium acetate, phenol red, sodium chloride, HEPES, and sodium pyruvate are also compatible ingredients in the weak acid-base subgroup. Derivatives of any of the compounds can also be employed in this group as long as such derivatives are compatible.

-24-

Riboflavin may also be placed in this group. Riboflavin is a photosensitizer and is the main nutrient responsible for the generation of hydrogen peroxides through the interaction with histidine, methionine, tyrosine and tryptophan (Griffin, F.M., et al., Cancer Research 91:2241-2248 (1981); Wang, R.J., et al., In Vitro 14:715-722 (1978)). By separating riboflavin from the amino acids, a stable concentrated subgroup is created.

A component that lends stability to liquid media subgroup comprising riboflavin is pyruvate. Pyruvate interacts with hydrogen peroxide to give acetic acid, carbon dioxide and water (Weil, L., Gordon, W.G., and Buchert, A.R., Arch. Biochem. Biophys. 33:90-109 (1951)). Since hydrogen peroxide is deleterious to cultured cells, membrane proteins, lipids and enzymes, controlling its build-up may be critical in a particular media. Thus, even if the desired media does not require pyruvate, the addition of pyruvate helps to increase the stability of the weak acid-base soluble subgroup and the shelf-life of the media prepared from this subgroup.

F. Alcohol Soluble Subgroup

Media formulations that contain cholesterol, steroids, tween 80, fat-soluble vitamins or other hydrophobic components may be concentrated into an alcohol-soluble group. Other hydrophobic compounds may be included in the alcohol-soluble group are well known to those of skill in the art.

Any combination of these components can be solubilized in a small volume of ethanol or propylene glycol. Once solubilized, these ingredients are then

-25-

microfluidized by well known techniques to form small lipid vesicles (Lidgate *et al.*, Bio Pharm 2(9): 28-33 (1989)). Briefly, the microfluidizer (Model 110 Microfluidizer) produces emulsions by combining shear, turbulence, and cavitation forces. Each cycle through the microfluidizer decreases the emulsion's mean droplet size ultimately producing submicron particles that are very stable. Water soluble components can also be trapped into these vesicles and then delivered to the culture media.

Fat soluble vitamins including vitamin A, D, E and K may also be included in this subgroup, as well as their derivatives. Steroids and their derivatives including cortisol, progesterone, estrogen, testosterone etc. are also included within the alcohol-soluble subgroup.

Fatty acids and lipids of the media formulation may be divided into this subgroup. Any fatty acid such as linoleic acid, oleic acid, arachidonic acid etc. fall into this classification. As will be appreciated, any lipid may also be included. Examples of lipids are lecithin, sphingomyelin and phospholipids. Lipids (oils) are usually necessary ingredients in this subgroup since microfluidization requires lipids (oils) to prepare lipid vesicles. In situations where the media formulation requires lipids but not the hydrophobic ingredients of this subgroup, it may be unnecessary to prepare an alcohol-soluble subgroup since such lipids can be included in the weak acid-base soluble group. In the weak acid-base soluble subgroup, lipids are desiccated onto sugars and/or salts.

-26-

Other media components that may be included in the alcohol-soluble group of the present invention may include tween 80 and 2-mercaptoethanol.

G. Alkali-Soluble Subgroups

For media components that require an alkaline environment for solubilization (pH 9.0), an alkali soluble subgroup is employed. This subgroup consists of the pyrimidines and purines that are stable and soluble at alkaline pH. These components, when dissolved in NaOH, form sodium salts which are soluble in an aqueous environment.

Pyrimidines and their derivatives that are compatible in this alkali subgroup include uracil and thymine. Xanthine and hypoxanthine are examples of purines that may also be include into the alkali soluble subgroup of the present invention. Other ingredients that are stable and soluble in this alkali environment are known by or easily determined by those of skill in the art.

H. Supplement-Containing Subgroup

As previously stated, typical medium formulations comprise amino acids, salts, vitamins, trace metals, sugars, lipids and nucleic acids. However, media are often supplemented with proteins, antibiotics or undefined broths/sera. When such a supplement is required for a particular medium, the supplement can be used directly (e.g., serum) or made into a concentrate (protein solutions such as insulin, transferrin and growth factors). The supplement-containing subgroup can then be combined

-27-

with the other concentrated subgroups to produce the desired media formulation.

A number of components that are included in the final media formulation, but not classified as a compatible ingredient in one of the aforementioned subgroups, may be added as one or more separate supplements. Thus, the present invention envisions the preparation of one or more supplement-containing subgroups that are not compatible in the above-noted subgroups. Such components that fall into the supplement subgroup include serum (fetal, horse, calf, etc.), yeastolate ultrafiltrate, tryptose phosphate broth, protein concentrates (insulin, transferrin, growth factors, hormones, albumin, etc.), antibiotics (gentamicin, penicillin, streptomycin, amphotericin B, etc.), whole egg ultrafiltrate, attachment factors (fibronectin, vitronectin, collagen, laminin, etc.) and NaHCO_3 liquid concentrate (for example, 25x). Depending on the final media formulation, one of ordinary skill in the art may select appropriate supplements which are compatible.

I. Kits

The subgroups of compatible ingredients of the invention are ideally suited for preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, test tubes, bottles, drums, and the like. Each of said container means comprises one of the individual subgroups of compatible ingredients as defined above. Such subgroups may be hydrated or dehydrated but are

typically a concentrated liquid solution. Such solutions may, according to the invention, be sterile.

A first container means may, for example, comprise any combination of media ingredients that are compatible in the acid-soluble subgroup. Compatible ingredients or any combination thereof that are included in the weak acid-base soluble group may be contained in a second container means. A third and fourth container means may comprise any combination of ingredients included in the alkali-soluble subgroup and alcohol-soluble subgroup, respectively. A fifth container means may comprise any combination of ingredients included in the glutamine-containing subgroup as long as divalent cations are included to stabilize the glutamine. A sixth container means may comprise ingredients listed in the supplement-containing subgroup. Other ingredients that are not compatible in the defined subgroups of the invention may be contained in one or more further container means to avoid mixing of incompatible components.

The number and types of subgroup containing container means comprising a kit for making a cell culture media may vary depending on the type of media to be prepared. Typically, the kit will contain the respective container means containing the subgroups necessary to make a particular media. However, additional subgroup containing container means may be included in the kit of the invention so that different media can be prepared by mixing different amounts of various subgroups to make different media formulations.

-29-

J. Mixing Concentrated Subgroups to Prepare a Culture Media

The media concentrate technology of the present invention provides a means to prepare one or more concentrated solutions of compatible ingredients by dividing these ingredients into the subgroups defined above. These subgroups, once prepared as concentrated solutions (greater than 1x), can be combined with a diluent to prepare the desired culture media. The amount of concentrated solution and amount of diluent needed may vary depending on the concentration of each subgroup, the number of subgroups and the desired concentration of the final media. One of ordinary skill in the art can easily determine a sufficient volume of a diluent and a sufficient volume of these concentrated solutions to prepare the desired media.

The pH of the resulting culture media can be adjusted by addition of acid or base. The media, however, may not require any adjustment, especially if the pH of each subgroup concentrate is adjusted so that the pH of the prepared media is in the desired range. Osmolarity of the media can also be adjusted after mixing the concentrated solutions with a diluent. Typically, the desired osmolarity may be predetermined and adjustments in the salt concentration of the concentrated solutions may be made to prepare a final media with the desired osmolarity.

K. Uses

The present invention provides concentrates that can easily be mixed in sufficient amounts with a diluent to prepare a culture media. Mixing these

-30-

concentrated subgroups can be done on a batch or continuous basis. In a batch system, appropriate amounts of the subgroup concentrates can be admixed with a diluent to prepare a single quantity of media. In a continuous system, sufficient amounts of each subgroup concentrate are continuously admixed with sufficient amounts of a diluent in a mixing chamber, while the culture media is continually removed. The obvious advantage of the continuous system is that a single reactor (mixing chamber) can be used to prepare media for a number of culture vessels or fermentation tanks. In addition, the continuous preparation of media in this manner can be accomplished in a closed sterile system.

According to the present invention, appropriate media concentrates can be prepared for any media and in any quantity. Such concentrates can thus be prepared and supplied commercially or made and used for in-house research. Customers that obtain prepared concentrates may prepare media in a more cost-effective manner. For example, the life of mixing tanks will be increased since certain corrosive dry powders need not be used to prepare media. Also, fewer man-hours may be needed to prepare the desired culture media.

Having now fully described the present invention, the same will be more clearly understood by reference to certain specific examples which are included herewith for purposes of illustration only, and not intended to be limiting of the invention, unless specified.

-31-

EXAMPLE I

Stabilization of Glutamine with Divalent Cations

L-Glutamine was admixed in a solution with varying amounts of calcium nitrate and magnesium sulfate at a pH of 5.5. Figure 1 shows that when the ratio of L-glutamine to divalent cations is significantly above 2.5:1, the stability of glutamine decreased. L-Glutamine was assayed by precolumn derivatization with O-phthaldialdehyde and then detected using high performance liquid chromatography (Rajendra, W. J. Liquid Chromat. 10(5):941-955 (1987)).

At a 2.5:1 glutamine to divalent cation ratio, glutamine remained stable in solution for 24 weeks. Cell culture media containing glutamine also contains divalent cations but due to the interaction of divalent cations with other amino acids present in the culture media, glutamine was not stabilized in 1x media formulation and begins to slowly degrade at 4°C with only 65-70% remaining after three weeks. (Sigma Cell Culture Reagents Catalogue (1991) pg. 202.)

EXAMPLE II

Preparation of RPMI-1640 Media

RPMI-1640 media is a media commonly used to grow mammalian cells in culture. The pH of this media is about 7.0 and the osmolarity can range from 265 to 300.

RPMI-1640 contains amino acids, including L-glutamine, various salts, vitamins, sugars, etc. The ingredients found in this media are described in

-32-

GIBCO/BRL Catalogue and Reference Guide, pg. 118, 1990.

The ingredients of RPMI-1640 media were divided into a number of compatible subgroups. Once the dry powder compatible ingredients were mixed, a concentrated solution of each subgroup was prepared. An appropriate volume of each of the prepared concentrated subgroups was then used to prepare a 1x RPMI-1640 media formulation.

Table 1 shows the ingredients of the RPMI-1640 media that were divided into the acid soluble subgroup. The concentrations correspond to a 1x formulation in 1 liter of solution. To make a 100x formulation of the acid soluble subgroup, the amount of each ingredient shown in Table 1 was added together to prepare a dry powder mix (DPM) of acid soluble ingredients. For example, 0.2 g of L-arginine, 0.05 g L-asparagine, 0.02 g L-aspartic acid, etc. were added to prepare a dry powder mix totaling 0.69 grams.

-33-

Table 1

Acid-Soluble Subgroup
for RPMI-1640 Media

Component	Molecular Weight	g/l	μM
L-Arginine	174	.200	1,149.43
L-Asparagine	132	.050	378.78
L-Aspartic Acid	133	.020	150.37
L-Cystine·2HCl	313	.06515	208.15
L-Glutamic Acid	147	.020	136.05
Glycine	75	.010	133.33
L-Histidine	155	.015	96.77
L-Hydroxyproline	131	.020	152.67
L-Isoleucine	131	.050	381.67
L-Leucine	131	.050	381.67
L-Lysine·HCl	183	.040	218.58
L-Methionine	149	.015	100.67
L-Phenylalanine	165	.015	89.82
L-Proline	115	.020	173.91
L-Serine	105	.030	285.71
L-Threonine	119	.020	168.07
L-Tryptophan	204	.005	24.51
L-Tyrosine	181	.02086	115.32
L-Valine	117	.020	170.94
Thiamine·HCl	337	.001	2.967

As shown in Table 2, the acid soluble compatible ingredients were mixed with 8.8 ml of water and then HCl was added to give a final pH of 0.85. This required about 1.2 ml of 5N HCl. The resulting

-34-

solution was a 100x formulation of the acid soluble subgroup for RPMI-1640 media.

Based on the 1640 media formulation, two additional subgroups were prepared: a glutamine-containing subgroup and a weak acid-base soluble subgroup. Table 3 shows the ingredients added to make a dry powder mix of the glutamine-containing subgroup for RPMI-1640 media. The mixture of ingredients totaling 6.4 g was mixed with about 17.5 ml water to prepare a 20 ml 50x concentrate with a final pH of about 5.5.

Table 4 shows the amount of ingredients needed to make 3.25 g of dry powder mix (DPM) for the weak acid-base soluble group. About 19.5 ml water was added to the weak acid-base ingredients to prepare a 20 ml solution with a pH of about 8.3. All ingredients in this subgroup were water soluble and therefore did not require desiccation onto a salt or sugar.

Reconstitution of the above-described acid-soluble subgroup, glutamine-containing subgroup and weak acid-base soluble subgroup was accomplished as shown in Table 2 to prepare a 1x RPMI-1640 media formulation. NaHCO_3 was added as a separate supplement. The osmolarity of the media was 276 mOsm before filter sterilization. Alternatively, a concentrated solution of NaHCO_3 can also be used rather than adding dry powder NaHCO_3 to the reconstituted media.

-35-

Table 2

Procedure for Preparation of RPMI-1640 Subgroups

1. Weak Acid-Base Soluble Subgroup (50X Concentrate)
Quantity sufficient to make 1 liter

19.5 ml water
3.25 g DPM

20.0 ml Total Volume Final pH = 8.28

Special Instructions: Add DPM slowly to water to avoid solidification of components. Use moderate stirring until dissolved (about 15-20 minutes).

2. Acid Soluble Subgroup (100X Concentrate)
Quantity sufficient to make 1 liter

8.8 ml water
0.69 g DPM
1.20 ml 5N HCl

10.0 ml Total Volume Final pH = 0.85

Special Instructions: Add 5N HCl slowly to the stirred solution.

3. Glutamine-Containing Subgroup (50X Concentrate)
Quantity sufficient to make 1 liter

17.5 ml water
6.4 g DPM

20.0 ml Total Volume Final pH = 5.50

Special Instructions: This is an endothermic process. The temperature of the solution will drop about 3°C with the addition of components. Use moderate stirring for about 10-15 minutes or until all components are dissolved.

-36-

Procedure for Preparing a 1X RPMI-1640 Media Formulation:

1. Add subgroup concentrates to water in the following order:

A.	950 ml water	pH 5.98
B.	20 ml Glutamine-Containing Subgroup	pH 5.61
C.	20 ml Weak Acid-Base Soluble Subgroup	pH 8.20
D.	10 ml Acid Soluble Subgroup	pH 4.91
E.	2.0 g NaHCO ₃	pH 7.00

Osmolarity before filtration: 276 m Osm

Table 3

Glutamine-Containing Subgroup
for RPMI-1640 Media

Component	Molecular Weight	g/l	μM
Ca(NO ₃) ₂	164.10	.0695	423.52
MgCl ₂	95.23	.0386	405.75
NaCl	58.44	6.000	102,669.4
L-Glutamine	146.00	.300	2,054.79
D-Ca Pantothenate	476.00	.0025	0.525

-37-

Table 4			
Weak Acid-Base Soluble Subgroup for RPMI-1640 Media			
Component	Molecular Weight	g/l	μ M
Biotin	244.00	.0002	.8196
Choline Chloride	140.00	.0030	21.4300
Folic Acid	441.00	.0010	2.2670
i-inositol	180.00	.0350	194.4400
Niacinamide	122.00	.0010	8.1960
Para-Aminobenzoic Acid	137.13	.0010	7.2900
Pyridoxine-HCl	206.00	.0010	4.8540
Riboflavin	376.00	.0002	.5320
Vitamin B12	1355.00	.000005	.00369
Glucose	180.16	2.0000	11,101.2400
Glutathione	307.33	.0010	3.2500
Phenol Red	376.36	.0050	13.2900
Na ₂ HPO ₄	141.96	.8000	5635.3900
KCl	74.56	.4000	5364.8100

EXAMPLE III

Reconstitution of RPMI-1640 Medium Concentrated Subgroups Using a Continuous Feed Mixing Chamber

A reconstitution experiment was carried out using RPMI-1640 media concentrate subgroups in the 15L Biospin bioreactor. Three media subgroups at 50X per subgroup and two 25X NaHCO₃ subgroup were continually pumped into the bioreactor at a rate of 250 ml/hour. Deionized distilled water was pumped into the reactor at a rate of 12.5L per hour. The perfused

-38-

concentrates were continually admixed in the mixing chamber. Complete 1X medium formulation was removed from the Biospin bioreactor at a rate of 12.5L per hour. The pH of the medium was monitored both internally and externally. Medium parameters (pH, osmolarity, amino acids, glucose, glutamine) were measured throughout the perfusion. The results of the study indicate that RPMI-1640 concentrated subgroups can be reconstituted in the Biospin bioreactor and the resulting 1X medium formulation parameters show significant correlation with the RPMI-1640 formulation (Figures 2,3 and Table 5).

-39-

Table 5
 Concentrate Reconstitution of RPMI 1640 Trial 1
 HPLC Assay Results

Amino Acid	Medium Theo.	Conc. Std.	Hour 1	Hour 3	Hour 5	Hour 6.5
Aspartate	20.00	20.30	21.00	20.93	20.77	19.47
Glutamate	20.00	20.84	23.91	22.46	21.05	19.95
Hydroxyproline	20.00	25.92	24.98	26.93	25.39	24.61
Asparagine	50.00	41.60	44.84	45.96	45.70	44.34
Arginine	200.00	192.03	199.53	197.26	198.54	195.83
Threonine	20.00	33.04	40.01	34.91	35.63	30.51
Proline	20.00	32.78	36.93	31.11	32.05	32.34
Tyrosine	20.00	25.84	26.63	26.77	24.81	19.11
Valine	20.00	22.15	22.90	22.66	21.83	18.40
Methionine	15.00	16.12	17.18	18.06	17.70	14.00
Cysteine	50.00	35.64	39.51	39.09	43.04	34.86
Isoleucine	50.00	56.71	53.72	51.85	51.38	49.54
Leucine	50.00	54.04	52.97	52.40	50.74	50.25
Phenylalanine	15.00	16.59	19.76	18.88	19.61	17.73
Tryptophan	5.00	4.66	7.67	7.62	7.99	6.47
Lysine	40.00	28.41	32.37	29.00	32.87	30.05
Histidine	15.000	15.000	14.414	13.899	12.884	10.540
Serine	30.000	30.000	31.030	30.399	30.745	22.250
Glycine	10.000	10.000	11.495	13.588	12.677	9.172

-40-

EXAMPLE IV

Preparation of DMEM Media

DMEM media (Dulbecco's Modified Eagle's Medium (GIBCO/BRL Catalogue and Reference Guide, pg. 96, 1990; Dulbecco, R. *et al.* *Virology* 8:396 (1959); and Smith, J.D. *et al.* *Virology* 12:185 (1960) was prepared by dividing the ingredients of this media into an acid-soluble subgroup, a glutamine-containing subgroup and a weak acid-base soluble subgroup. The ingredients of DMEM media were divided and prepared as a concentrated subgroups as shown in Tables 6, 7, 8 and 9. A procedure similar to that described in Example II was used.

Table 9 also shows the procedure used for mixing and diluting the DMEM subgroups to make a 1x formulation of DMEM. NaOH was added to the 1x media to adjust the final pH to 7.3. NaHCO₃ was added as a buffering agent.

-41-

Table 6
Acid Soluble Subgroup for DMEM Media

Components	MW	g/L	uM
L-Arginine.HCl	210.66	0.084	398.7
L-Cystine.2HCL	313.22	0.06257	199.8
L-Glycine	75.0	0.030	400.0
L-Histidine.HCl.H ₂ O	209.46	0.042	200.5
L-Isoleucine	131.0	0.105	801.5
L-Leucine	131.0	0.105	801.5
L-Lysine.HCl	182.46	0.146	800.2
L-Methionine	149.0	0.030	201.3
L-Phenylalanine	165.0	0.066	400.0
L-Serine	105.0	0.042	400.0
L-Threonine	119.0	0.095	798.3
L-Tryptophan	204.0	0.016	78.4
L-Tyrosine	181.0	0.072	397.8
L-Valine	117.0	0.094	803.4
NaH ₂ PO ₄ .H ₂ O	137.98	0.125	905.9
Thiamine.HCl	337.28	0.004	11.9
FeCl ₃ .6H ₂ O	270.22	0.000054	0.2

-42-

Table 7
Glutamine-Containing Subgroup
for DMEM Media

Components	MW	g/L	uM
CaCl ₂ (anhyd)	110.99	0.200	1802.0
NaCl	58.45	5.400	92386.7
MgCl ₂ (anhyd)	95.23	0.07726	811.3
D-Ca Pantothenate	476.53	0.004	8.4
L-Glutamine	146.15	0.584	3995.9

Table 8
Weak Acid-Base Soluble Solution
for DMEM Media

Components	MW	g/L	uM
NaCl	58.45	0.400	6843.5
Glucose	180.16	4.500	24977.8
Na-Pyruvate	111.05	0.110	990.5
Choline Chloride	140.0	0.004	28.6
Folic Acid	441.0	0.004	9.1
Inositol	180.0	0.0072	40.0
Niacinamide	122.0	0.004	32.8
Pyridoxal.HCl	203.62	0.004	19.6
Riboflavin	376.0	0.0004	1.1
KCl	74.56	0.400	5364.8

Table 9
Procedure for Preparation of DMEM Subgroups

1. DMEM High Glucose
Glutamine-Containing Subgroup

18.1 ml H₂O
6.3 g DPM

20.0 ml Total Volume pH 5.50 5 0 X
Concentrate

-43-

2. DMEM High Glucose
Weak Acid-Base Soluble Subgroup

17.0 ml H₂O
5.4 g DPM
0.7 ml 0.1N NaOH

20.0 ml Total Volume pH 6.60 5 0 X
Concentrate

3. DMEM High Glucose
Acid Soluble Subgroup

18.5 ml H₂O
1.1 g DPM
1.5 ml 5N HCl

20.0 ml Total Volume pH 0.80 5 0 X
Concentrate

Procedure for Preparing a 1X DMEM Media
Formulation

900.0 ml H₂O
20.0 ml Glutamine-Containing Subgroup
20.0 ml Weak Acid-Base Subgroup
20.0 ml Acid Soluble Subgroup
3.7 g NaHCO₃
ADD 5N NaOH to bring pH to 7.30
ADD H₂O to bring volume to 1000.0 ml

1000.0 ml Total Volume pH 7.30 1X Formulation

Osmolarity will be 315-340 mOsm. Osmolarity will change with the amount of acid/base used for the solubilization of components in each subgroup. If the osmolarity falls above the desired limits an adjustment in the NaCl concentration can be made to lower the osmolarity.

EXAMPLE V

Preparation of HAM's F-12 Medium

HAM's F-12 medium (Ham, R.G., Proc. Natl. Acad. Sci. 53:288 (1965)) is prepared by dividing the ingredients of this media into an acid-soluble

-44-

subgroup, a glutamine-containing subgroup, and a weak acid/base subgroup. The compositions of the respective subgroups are set out in Tables 10, 11 and 12. A procedure similar to that described in Example II may be used to prepare the media concentrates.

-45-

Table 10
ACID SOLUBLE SUBGROUP FOR HAM'S F-12 MEDIUM

COMPONENT	mg/L
L-ALANINE	8.90
L-ARGININE.HCL	211.00
L-ASPARAGINE.H ₂ O	15.01
L-ASPARTIC ACID	13.30
L-CYSTEINE. HCL.H ₂ O	35.12
L-GLUTAMIC ACID	14.70
GLYCINE	7.50
L-HISTIDINE.HCL.H ₂ O	20.96
L-ISOLEUCINE	3.94
L-LEUCINE	13.10
L-LYSINE.HCL	36.50
L-METHIONINE	4.48
L-PHENYLALANINE	4.96
L-PROLINE	34.50
L-SERINE	10.50
L-THREONINE	11.90
L-TRYPTOPHAN	2.04
L-TYROSINE	5.40
L-VALINE	11.70
THIAMINE.HCL	0.34
FeSO ₄ .7H ₂ O	0.834

-46-

Table 11

GLUTAMINE CONTAINING SUBGROUP FROM HAM'S F-12 MEDIUM

COMPONENT	mg/L
L-GLUTAMINE	146.00
D-CA PANTOTHENATE	0.48
CaCl ₂ (anhyd)	33.22
MgCl ₂ (anhyd)	57.22
NaCl	6000.00

-47-

Table 12
WEAK ACID/BASE SUBGROUP FOR HAM'S F-12 MEDIUM

COMPONENT	mg/L
BIOTIN	0.0073
CHOLINE CHLORIDE	13.9600
FOLIC ACID	1.3000
i-INOSITOL	18.0000
NIACINAMIDE	0.0370
PYRIDOXINE.HCL	0.0620
RIBOFLAVIN	0.0380
VITAMINE B ₁₂	1.3600
D-GLUCOSE	1802.0000
HYPOXANTHINE (sodium salt)	4.7700
LINOLEIC ACID	0.0840
LIPOIC ACID	0.2100
PHENOL RED	1.2000
PUTRESCINE.2HCL	0.1610
SODIUM PYRUVATE	110.0000
THYMIDINE	0.7300
CuSO ₄ .5H ₂ O	0.0025
KCL	223.6000
NaCl	1599.0000
Na ₂ HPO ₄ (anhyd)	142.0400
ZnSO ₄ .7H ₂ O	0.8630

-48-

EXAMPLE VI

Preparation of DMEM/F-12 Medium

DMEM/F-12 medium (GIBCO/BRL Catalogue and Reference Guide, page 97 (1990)) is prepared by dividing the ingredients of this media into an acid-soluble subgroup, a glutamine-containing subgroup, and a weak acid/base subgroup. The compositions of the respective subgroups are set out in Tables 13, 14 and 15. A procedure similar to that described in Example II may be used to prepare the media concentrates.

-49-

Table 13
ACID SOLUBLE SUBGROUP FOR DMEM/ F-12 MEDIUM

COMPONENT	mg/L
L-ALANINE	4.45
L-ARGININE.HCL	147.50
L-ASPARAGINE.H ₂ O	7.50
L-ASPARTIC ACID	6.65
L-CYSTEINE.HCL.H ₂ O	17.56
L-GLUTAMIC ACID	7.35
GLYCINE	18.75
L-HISTIDINE.HCL.H ₂ O	31.48
L-ISOLEUCINE	54.47
L-LEUCINE	59.05
L-LYSINE.HCL	91.25
L-METHIONINE	17.24
L-PHENYLALANINE	35.48
L-PROLINE	17.25
L-SERINE	26.25
L-THREONINE	53.45
L-TRYPTOPHAN	9.02
L-TYROSINE	38.39
L-VALINE	52.85
THIAMINE.HCL	2.17
FeSO ₄ .7H ₂ O	0.417
NaH ₂ PO ₄ .H ₂ O	42.50
L-CYSTINE.2HCL	31.29
Fe(NO ₃) ₂ .9H ₂ O	0.05

-50-

Table 14

WEAK ACID/BASE SUBGROUP FOR DMEM/ F-12 MEDIUM

COMPONENT	mg/L
BIOTIN	0.0035
CHOLINE CHLORIDE	8.9800
FOLIC ACID	2.6500
i-INOSITOL	12.6000
NIACINAMIDE	2.0200
PYRIDOXINE.HCL	0.0310
RIBOFLAVIN	0.2190
VITAMINE B ₁₂	0.0680
D-GLUCOSE	3151.0000
HYPOXANTHINE (sodium salt)	2.3900
LINOLEIC ACID	0.0420
LIPOIC ACID	0.1050
PHENOL RED	8.1000
PUTRESCINE.2HCL	0.0810
SODIUM PYRUVATE	55.0000
THYMIDINE	0.3650
CuSO ₄ .5H ₂ O	0.0013
KCL	311.8000
NaCL	999.5000
Na ₂ HPO ₄ (anhyd)	71.02
ZnSO ₄ .7H ₂ O	0.4320
NaH ₂ PO ₄ .H ₂ O	20.0000
PYRIDOXAL.HCL	2.0000
HEPES	3574.5000

-51-

Table 15

GLUTAMINE CONTAINING SUBGROUP FOR DMEM/ F-12 MEDIUM

COMPONENT	mg/L
L-GLUTAMINE	365.00
D-CA PANTOTHENATE	2.24
CaCl ₂ (anhyd)	116.60
MgCl ₂ (anhyd)	28.64
MgSO ₄	48.84
NaCl	6000.00

EXAMPLE VII

Preparation of IMDM Medium

IMDM medium (Dulbecco, R. and Freeman, G., Virology 8:396 (1959); Smith, J.D. et al., Virology 12:185 (1960); Tissue Culture Standards Committee, In Vitro 6:2,93; In Vitro 9:6 (1970); Iscove, N.N. and Melchers, F., J. Exp. Med. 147:923) is prepared by dividing the ingredients of this media into an acid-soluble subgroup, a glutamine-containing subgroup, and a weak acid/base subgroup. The compositions of the respective subgroups are set out in Tables 16, 17 and 18. A procedure similar to that described in Example II may be used to prepare the media concentrates.

-52-

Table 16
ACID SOLUBLE SUBGROUP FOR IMDM MEDIUM

COMPONENT	mg/L
L-ALANINE	25.00
L-ARGININE.HCL	84.00
L-ASPARAGINE.H ₂ O	84.00
L-ASPARTIC ACID	30.00
L-CYSTINE.2HCL	91.24
L-GLUTAMIC ACID	75.00
GLYCINE	30.00
L-HISTIDINE.HCL.H ₂ O	42.00
L-ISOLEUCINE	105.00
L-LEUCINE	105.00
L-LYSINE.HCL	146.00
L-METHIONINE	30.00
L-PHENYLALANINE	66.00
L-PROLINE	40.00
L-SERINE	42.00
L-THREONINE	95.00
L-TRYPTOPHAN	16.00
L-TYROSINE	71.43
L-VALINE	94.00
THIAMINE.HCL	4.00
NaH ₂ PO ₄ .H ₂ O	125.00

-53-

Table 17
WEAK ACID/BASE SUBGROUP FOR IMDM MEDIUM

COMPONENT	mg/L
BIOTIN	0.0130
CHOLINE CHLORIDE	4.0000
FOLIC ACID	4.0000
i-1NOSITOL	7.2000
NIACINAMIDE	4.0000
PYRIDOXAL.HCL	4.0000
RIBOFLAVIN	0.4000
VITAMINE B ₁₂	0.0130
D-GLUCOSE	4500.000
HEPES	5958.0000
PHENOL RED	15.0000
SODIUM PYRUVATE	110.0000
KCL	330.0000
KNO ₃	0.0760
NaSeO ₃ 5H ₂ O	0.0173

Table 18
GLUTAMINE CONTAINING SUBGROUP FOR IMDM MEDIUM

COMPONENT	mg/L
L-GLUTAMINE	584.00
D-CA PANTOTHENATE	4.00
CaCl ₂ (anhyd)	165.0
MgSO ₄ (anhyd)	97.67
NaCl	4505.00

-54-

Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the media and fermentation art and/or related fields are intended to be within the scope of the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practice within the scope of the appended claims.

What Is Claimed Is:

1. A method of preparing a cell culture media comprising

(a) dividing the ingredients of a cell culture media into one or more compatible subgroups, said compatible subgroups comprising:

- i. an acid soluble subgroup,
- ii. a weak acid-base soluble subgroup,
- iii. a glutamine-containing subgroup,
- iv. an alcohol soluble subgroup,
- v. an alkali soluble subgroup, and
- vi. a supplement-containing subgroup;

and

(b) admixing a sufficient amount of each of said subgroups obtained in step (a) with a sufficient amount of a diluent to give said cell culture media.

2. A method of preparing a cell culture media comprising

(a) dividing the ingredients of a cell culture media into one or more compatible subgroups, said compatible groups comprising

- i. an acid soluble subgroup,
- ii. a weak acid-base soluble subgroup,
- iii. a glutamine-containing subgroup,
- iv. an alcohol-soluble subgroup,
- v. an alkali-soluble subgroup, and
- vi. a supplement-containing subgroup;

(b) dissolving each of said subgroups in a carrier to give concentrated solutions of said subgroups in the respective carrier; and

(c) admixing a sufficient amount of each of said subgroups obtained in step (b) with a sufficient amount of a diluent to give said cell culture media.

3. The method of any one of claims 1 or 2, wherein the cell culture media is a 10x media formulation.

4. The method of any one of claims 1 or 2, wherein the cell culture media is a 1x media formulation.

5. The method of any one of claims 1 or 2, wherein the cell culture media is a RPMI-1640 media.

6. The method of any one of claims 1 or 2, wherein the cell culture media is a DMEM media.

7. The method of claim 2, wherein said admixing is accomplished in a batch system.

8. The method of claim 2, wherein said admixing is accomplished in a continuous system.

9. The method of claim 2, wherein the concentrated solutions obtained in step (b) are greater than 10x.

10. The method of claim 2, wherein the concentrated solution obtained in step (b) is about 50x.

-57-

11. The method of claim 2, wherein the concentrated solution obtained in step (b) is about 100x.

12. The method of any one of claims 1 or 2, wherein the acid-soluble subgroup comprises ingredients which are stable and soluble at a pH of about 1.

13. The method of claim 12, wherein said subgroup comprises one or more ingredients selected from the group consisting of thiamine, ascorbic acid, a ferric salt, a ferrous salt, a phosphate and amino acids other than glutamine.

14. The method of any one of claims 1 or 2, wherein the weak acid-base soluble subgroup comprises ingredients which are stable and soluble at a pH of about 4 to 9.

15. The method of claim 14, wherein said acid-base soluble subgroup comprises one or more ingredients selected from the group consisting of a sugar, a deoxyribose, a ribose, a nucleotide, a trace element, a lipid, phenol red, sodium pyruvate, HEPES, Na_2HPO_4 , NaCl , KCl , sodium acetate and a water-soluble vitamin other than a thiamine, ascorbic acid or D-Ca pantothenate.

16. The method of any one of claims 1 or 2, wherein the glutamine containing subgroup comprises glutamine and at least one divalent cation in an amount effective to stabilize glutamine.

-58-

17. The method of claim 16, wherein said divalent cation is calcium, magnesium, or manganese, or a combination thereof.

18. The method of claim 16, wherein the subgroup further comprises sodium chloride, or D-Ca pantothenate or combinations thereof.

19. The method of any one of claims 1 or 2, wherein the alcohol soluble subgroup comprises one or more ingredients selected from the group consisting of cholesterol, linoleic acid, lipoic acid, a fat soluble vitamin, a menadione, 2-mercaptoethanol, a lipid, and a fatty acid.

20. The method of any one of claims 1 or 2, wherein the alkali soluble subgroup comprises one or more ingredients which are soluble and stable at a pH of about 9.0.

21. The method of claim 20, wherein said alkali soluble subgroup comprises one or more ingredients selected from the group consisting of a uracil, a xanthine and a hypoxanthine.

22. A method for preparing a cell culture medium, comprising admixing a sufficient amount of an aqueous diluent with a sufficient amount of at least one subgroup of ingredients dissolved in a carrier comprising:

- i. an acid soluble subgroup,
- ii. a weak acid-base soluble subgroup,
- iii. a glutamine-containing subgroup,
- iv. an alcohol-soluble subgroup,

-59-

v. an alkali-soluble subgroup, and
vi. a supplement-containing subgroup,
to give said cell culture media.

23. The method of claim 22, wherein said admixing is accomplished in a batch system.

24. The method of claim 22, wherein said admixing is accomplished in a continuous system.

25. A method of preparing RPMI-1640 media comprising

(a) dividing the ingredients of RPMI-1640 media into subgroups, said subgroups comprising

i. an acid soluble subgroup,
ii. a weak acid-base soluble subgroup, and

iii. a glutamine-containing subgroup;

(b) dissolving each of the subgroups of compatible ingredients in a carrier to give concentrated solutions of the subgroups in the respective carrier; and

(c) admixing a sufficient amount of each of the subgroups obtained in step (b) with a sufficient amount of a diluent to produce the RPMI-1640 media.

26. A method of preparing DMEM media comprising

(a) dividing the ingredients of DMEM media into subgroups, said subgroups comprising

i. an acid soluble subgroup,
ii. a weak acid-base soluble subgroup, and
iii. a glutamine-containing subgroup;

-60-

(b) dissolving each of the subgroups of compatible ingredients in a carrier to give concentrated solutions of the subgroups in the respective carrier; and

(c) admixing a sufficient amount of each of the subgroups obtained in step (b) with a sufficient amount of a diluent to produce the DMEM media.

27. A kit for making a cell culture media, wherein said kit comprises a carrier being compartmentalized to receive in close confinement one or more containers wherein

- (a) a first container means contains compatible ingredients of an acid soluble subgroup;
- (b) a second container means contains compatible ingredients of a weak acid-base soluble subgroup;
- (c) a third container means contains compatible ingredients of a glutamine-containing subgroup;
- (d) a fourth container means contains compatible ingredients of an alkali soluble subgroup;
- (e) a fifth container means contains compatible ingredients of an alcohol soluble subgroup; and
- (f) a sixth container means contains compatible ingredients of a supplement-containing subgroup.

28. The kit of claim 27, wherein the cell culture media is a RPMI-1640 media.

-61-

29. The kit of claim 27, wherein the cell culture media is a DMEM media.

30. The kit of claim 27, wherein said ingredients in at least one of said container means is in solution.

31. The kit of claim 30, wherein said solution is a concentrated solution of said compatible ingredients.

32. The kit of claim 31, wherein said concentrated solution is about 25x.

33. The kit of claim 31, wherein said concentrated solution is about 50x.

34. The kit of claim 31, wherein said concentrated solution is sterile.

35. A composition of matter comprising glutamine and a divalent cation in an amount effective to stabilize glutamine.

36. The composition of matter of claim 35, wherein the glutamine and divalent cation are in solution.

37. The composition of matter of claim 36, wherein the ratio of glutamine to divalent cation is about 2:1.

-62-

38. The composition of matter of claim 36, wherein said divalent cation comprises a calcium salt, a magnesium salt or a combination thereof.

39. The composition of matter of claim 36, wherein said solution is sterile.

40. A kit for making RPMI-1640 media, wherein said kit comprises a carrier being compartmentalized to receive in close confinement therein:

(a) a first containing means containing compatible ingredients of an acid soluble subgroup;

(b) a second container means containing compatible ingredients of a weak acid-base soluble subgroup; and

(c) a third container means containing compatible ingredients of a glutamine-containing subgroup.

41. A kit for making DMEM media, wherein said kit comprises a carrier being compartmentalized to receive in close confinement therein:

(a) a first containing means containing compatible ingredients of an acid soluble subgroup;

(b) a second container means containing compatible ingredients of a weak acid-base soluble subgroup; and

(c) a third container means containing compatible ingredients of a glutamine-containing subgroup.

1/4

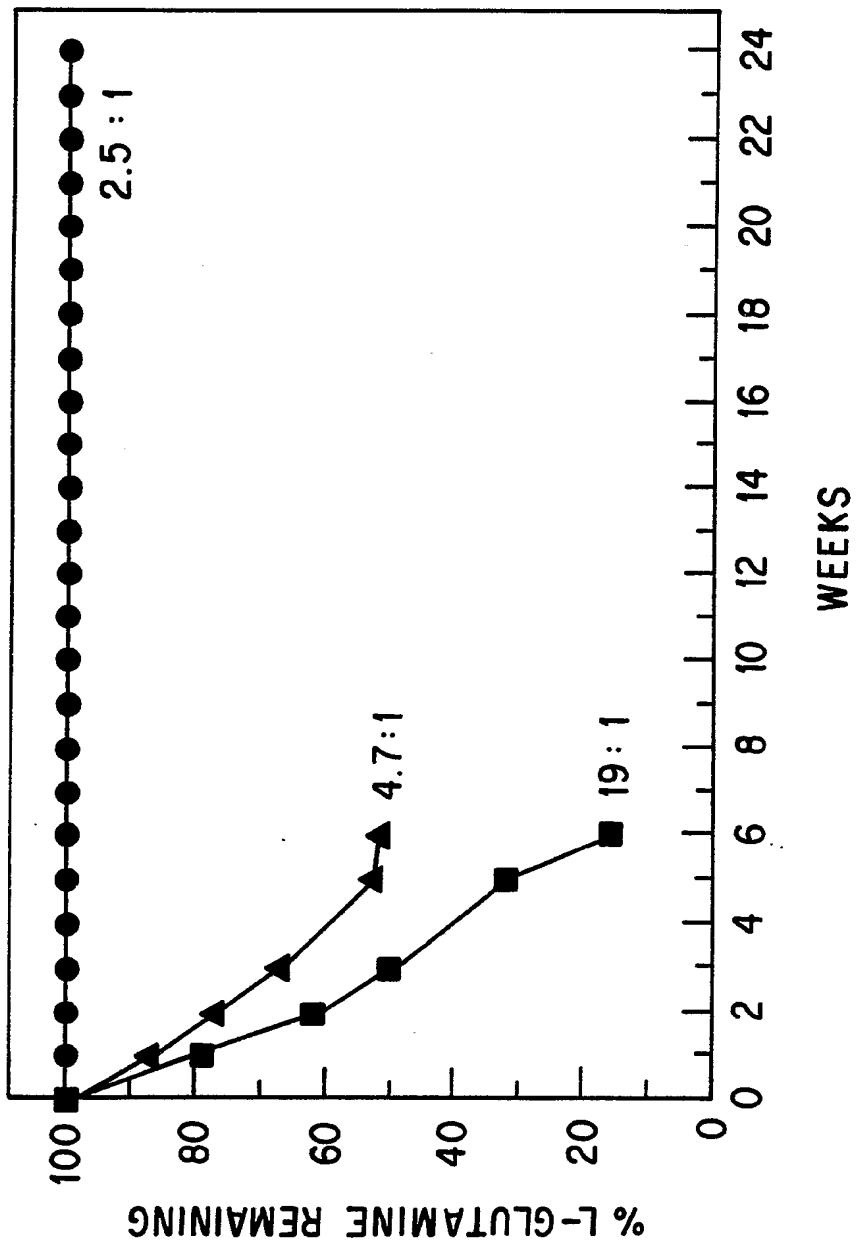


FIG. 1

2/4

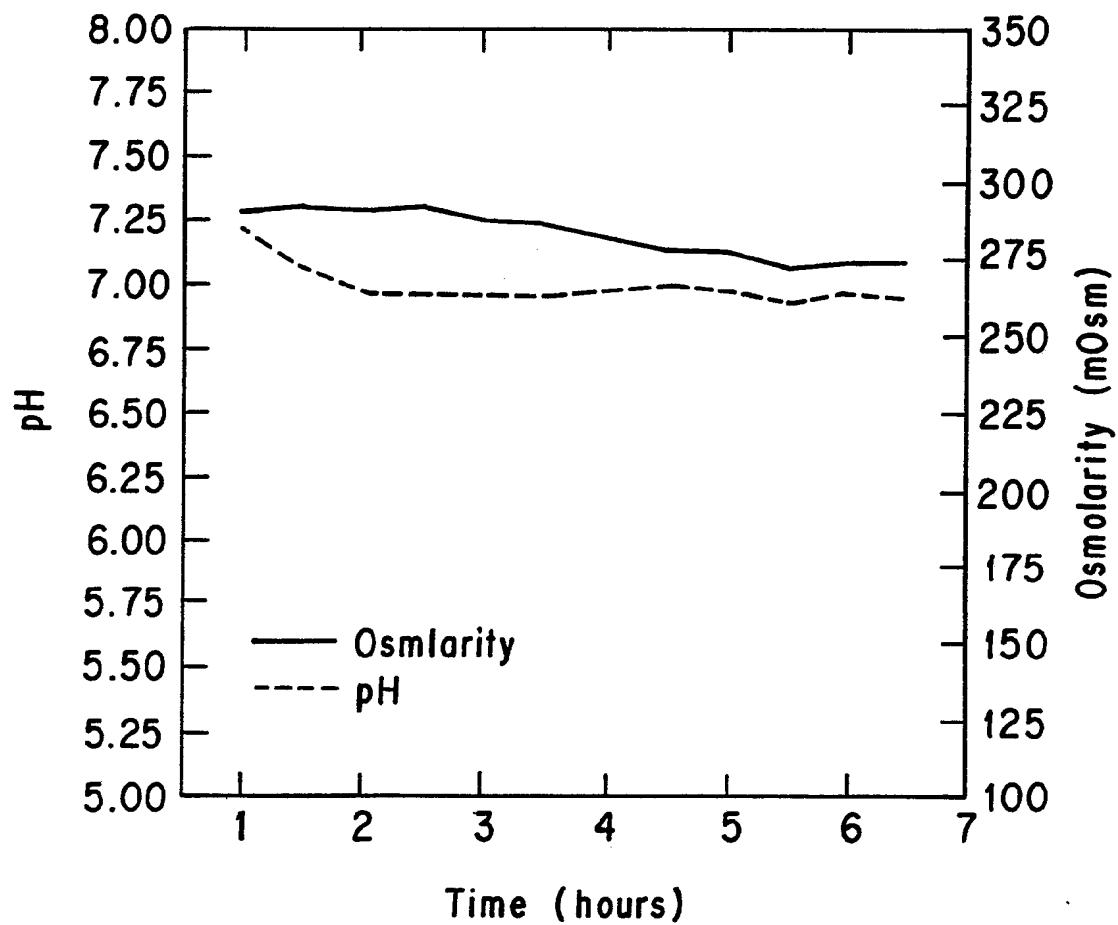


FIG. 2

3/4

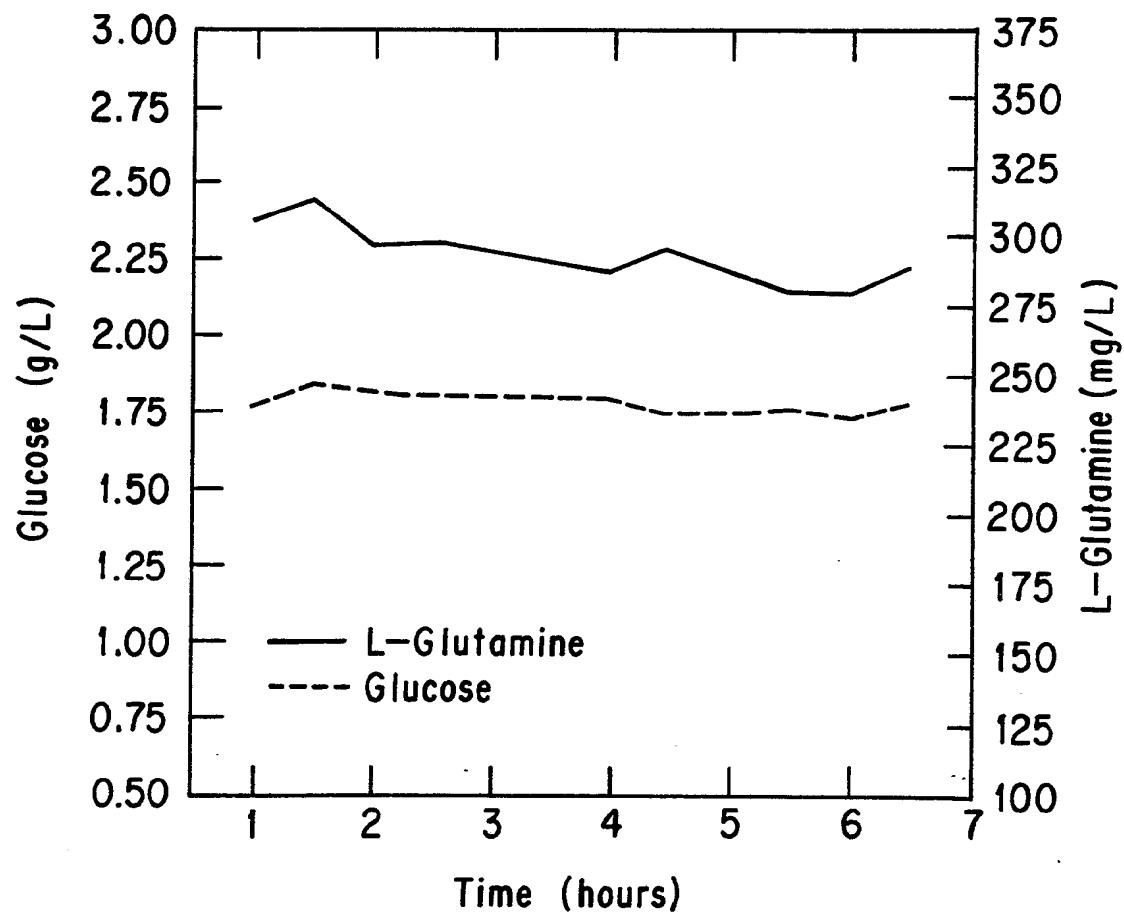


FIG. 3

4/4

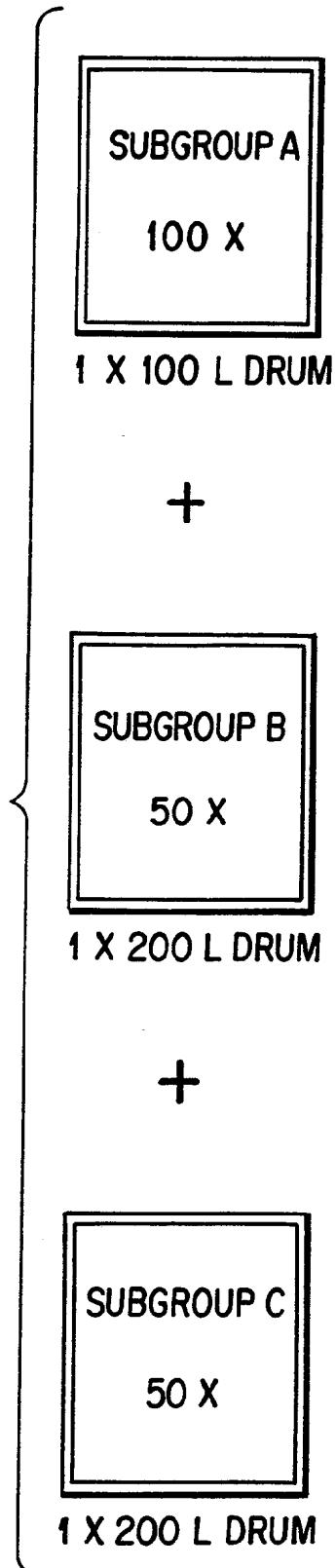


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/04988

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :IPC (5) C12N 5/00
 US CL :435/240.54, 240.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.54, 240.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Online, BIOSIS
 search terms: glutamine, divalent metal cation, cation, media, calcium, stock

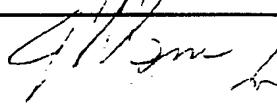
C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	U.S.A, 4,879,222 (ALDERMAN ET AL) 07 November 1989, see entire document.	35-39
Y	Biotechnological Progress, Volume 6, issued 1990, S.S. Ozturk et al, "Chemical Decomposition of Glutamine in Cell Culture Media: Effect of Media Type, pH, and Serum Concentration, pages 121-128, see entire document	1-41
Y	"Methods for Preparation of Media, Supplements, and Substrata for Serum-Free Animal Cell Culture," published 1984 by Alan R. Liss, (NY) see pages 23-68	1-41

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance		
E earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*&*	document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
07 AUGUST 1992	17 SEP 1992

Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer
Facsimile No. NOT APPLICABLE	JANE WILLIAMS  Telephone No. (703) 308-0196